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# **Dynamic Compound-Specific Stable Isotope Probing of the Soil Microbial Biomass**

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## Introduction

The concept of following the assimilation of stable isotope labelled substrates into newly synthesised soil microbial biomass biochemicals (= biomarkers) was first demonstrated nearly 20 years ago in studies of unculturable soil methanotrophs [Radajewski *et al.*, 2000; Bull *et al.*, 2000]. These landmark papers laid the foundations for an approach that has come to be generally known as stable isotope probing (SIP). The major advantage of SIP in studying the soil microbial biomass lies in its effectiveness for investigating unculturable soil microorganisms, i.e. the vast majority of the soil bacteria, actinomycetes, fungi, algae and protozoa. Historically, SIP has developed along two distinct paths, namely those applications focussing on genomic material (DNA and RNA) and those targeting small molecules, such as phospholipid fatty acids (PLFAs).

The basic soil SIP experiment involves presenting the microbial community with a stable isotopically-labelled substrate, most commonly  $^{13}\text{C}$ -labelled. After a period of incubation soils are sampled and specific cellular biochemicals extracted. The increase in weight associated with uptake of the stable isotope label is utilised to either separate the labelled components gravimetrically (e.g. DNA and RNA) or analyse directly by isotope ratio mass spectrometry (IRMS). The original experiment performed by Boschker and co-workers traced  $^{13}\text{C}$ -labelled acetate utilisation in aquatic sediments [Boschker *et al.*, 1998], with the resulting phospholipids being solvent extracted, hydrolysed and the fatty acids (PLFAs) analysed by gas chromatography-combustion-IRMS (GC-C-IRMS) to determine the pattern of uptake of the  $^{13}\text{C}$ -label into different components – the distribution of the stable isotopically-labelled PLFAs was used as a taxonomic fingerprint for the microbes assimilating different substrates. In the original experiment  $^{13}\text{C}$  from acetate was recovered mainly from PLFAs with even numbers of carbon atoms (16:1 $\omega$ 7c, 16:0, 18:1 $\omega$ 7c), whereas fatty acids with odd numbers of carbon atoms (a15:0, 15:0, 17:1 $\omega$ 6, 17:0) were primarily labelled upon incubation with propionate. Comparison of the  $^{13}\text{C}$ -PLFA compositions with those of known microorganisms, showed that the two substrates were predominantly consumed by different specialized groups of sulphate-reducing bacteria (SRB). The PLFA  $^{13}\text{C}$ -labelling pattern for the acetate consumers was similar to *Desulfotomaculum acetoxidans* and *Desulfofrigus* spp., two acetate-consuming SRB, while those of the propionate consumers did not resemble any known strain. Despite the obvious effectiveness of this experiment one of the criticisms of PLFA-SIP has been the lack of specificity in its ability to taxonomically identify specific microorganisms. We will show in this contribution, however, that such criticism overlooks how the use of specific  $^{13}\text{C}$  or  $^{15}\text{N}$ -labelled tracers together with GC-C-IRMS analysis of different classes of small molecules, can be extremely effective in exploring critical aspects of soil biomass characteristics, which extend well beyond taxonomic assignments.

DNA- and RNA-SIP are without question the preferred techniques for identifying active microorganisms assimilating particular carbon substrates and nutrients into cellular biomass. The

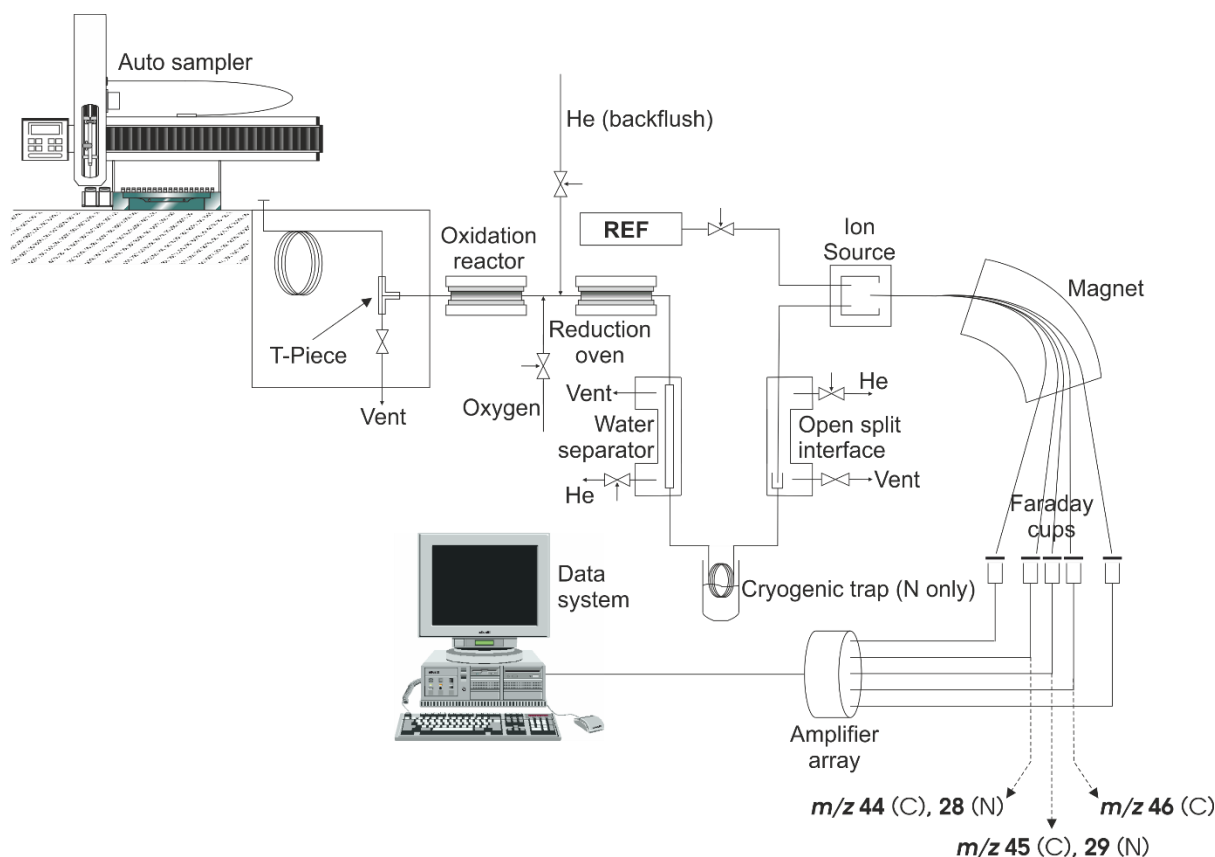
initial demonstration of this approach immediately showed its value as a cultivation-independent technique for linking metabolic function to specific members of microbial communities inhabiting complex environmental matrices, such as soils. As with PLFA-SIP, soils are incubated with stable-isotope labelled substrates, but the technique departs from the PLFA approach since extracted nucleic acids are subjected to density gradient ultracentrifugation to separate nucleic acids of differing masses due to  $^{13}\text{C}$ -labelling. Purification of DNA from caesium chloride retrieves  $^{13}\text{C}$ -labelled and unlabelled DNA (differentiated by IRMS), followed by polymerase chain reaction (PCR) amplification and molecular characterization (e.g. fingerprinting, microarrays, clone libraries, metagenomics). The approach has proved to be extremely powerful in a wide variety of environmental settings. For the procedure to succeed, however, a very high level of  $^{13}\text{C}$ -labelling is required to ensure that physical separation is achieved during the centrifugation step. Even higher levels of labelling are required for  $^{15}\text{N}$ -DNA/RNA SIP due to the lower molar ratios of N/C.

We do not dwell further in this contribution on the use of DNA/RNA-SIP approaches to soil biomass studies. Instead we focus on exploring an under-appreciated dimension of small molecule SIP, which we now term *dynamic-SIP*. This is an approach we have come to use routinely in our laboratory over the past ten years. We will focus initially on dynamic  $^{13}\text{C}$ -SIP then move to  $^{15}\text{N}$ - and dual  $^{13}\text{C}$ ,  $^{15}\text{N}$ -SIP. Overall, we will demonstrate how the small molecule SIP technique has progressed from the original and widely used  $^{13}\text{C}$ -PLFA taxonomic approach to a significantly more powerful tool for exploring soil biomass dynamics in relation to: (i) revealing the assimilation of different substrates, (ii) the turnover rates of different biomass C pools, (iii) the connectivity between C and N cycles, and (iv) trophic interactions. This contribution can be viewed as an update to our previous reviews of  $^{13}\text{C}$ -PLFA SIP applied to soils [Evershed *et al.*, 2006, 2007; Maxfield and Evershed, 2011], but with emphasis on the dynamic-SIP approach. Our aim here is to demonstrate how small molecule dynamic-SIP is much more than a taxonomic technique, yielding information generally inaccessible to genomic and bulk stable isotope approaches.

### **General considerations for stable isotope probing in the soil system**

SIP in various forms has been applied in investigations of microbial communities in a diversity of environments ranging from ocean sediments to the human gut, but with soil SIP arguably being the most challenging of all the systems studied [Murrell and Whiteley, 2011].  $^{13}\text{C}$ -SIP is far more commonly used than any other isotope system, with substrates used in soil SIP studies including:  $^{13}\text{CH}_4$ ,  $^{13}\text{CO}_2$ ,  $^{13}\text{CH}_3\text{OH}$ ,  $^{13}\text{C}$ -acetate,  $^{13}\text{C}$ -propionate,  $^{13}\text{C}$ -butyrate,  $^{13}\text{C}$ -pyruvate,  $^{13}\text{C}$ -mono- and polysaccharides,  $^{13}\text{C}$ -alkyl halides,  $^{13}\text{C}$ -aromatics (including phenolic and PAHs) and complex substrates such as  $^{13}\text{C}$ -labelled plants or cultured microbes. After incubation the vast majority of studies target either DNA/RNA or PLFAs. The major advantages of  $^{13}\text{C}$ -PLFA SIP are that the preparation of PLFAs from soils is entirely routine (see complete Experimental Methods at the end of

this chapter) combined with the exceptional sensitivity of the final analysis [Mottram and Evershed, 2003], which rests upon the use of GC-C-IRMS (Fig. 1).



**Figure 1.** The instrument that made small molecule SIP possible [Merritt and Hayes, 1994]. A GC-C-IRMS configured for either compound-specific carbon or nitrogen isotope analyses. For  $\delta^{13}\text{C}$  value determinations ions at  $m/z$  44 ( $^{13}\text{C}^{16}\text{O}^{16}\text{O}$ ), 45 ( $^{13}\text{C}^{16}\text{O}^{16}\text{O} + ^{12}\text{C}^{16}\text{O}^{17}\text{O}$ ) and 46 ( $^{13}\text{C}^{16}\text{O}^{17}\text{O} + ^{12}\text{C}^{16}\text{O}^{18}\text{O} + ^{12}\text{C}^{17}\text{O}^{17}\text{O}$ ) are continuously measured; monitoring  $m/z$  46 enables the  $^{17}\text{O}$  (via determination of  $^{18}\text{O}$  content) contribution to  $m/z$  45 to be accounted for. For  $\delta^{15}\text{N}$  determinations ions at  $m/z$  28 ( $^{14}\text{N}^{14}\text{N}$ ) and 29 ( $^{14}\text{N}^{15}\text{N}$ ) are monitored. The exact derivatisation, standardisation and calibration, GC columns and temperature programmes and data analysis protocols are given at the end of this chapter for all the compound classes referred to in the following sections. Further detailed discussions of the technical aspects of the instrumentation in the context of ecological research are given in Evershed *et al.* [2007].

Indeed, it is the sensitivity aspect, combined with the selectivity conferred through the choice of  $^{13}\text{C}$ -labelled substrate that makes the  $^{13}\text{C}$ -PLFA SIP such a powerful tool in studying the soil microbial biomass. The high sensitivity of the technique has a number of important practical advantages: (i) economics – stable isotopically- labelled substrates are amongst the most expensive chemicals used in ecological research. The high sensitivity of the GC-C-IRMS means that less isotope ( $^{13}\text{C}$ ,  $^{15}\text{N}$ ) is required per treatment which has obvious cost savings. This has critical advantages in terms of improved experimental design as scale can be increased, i.e. increased replication or more frequent sampling in time-course experiments, which amounts to enhanced rigour and ultimately greater insights into biomass function, (ii) appropriate application rates – this is critical to avoid perturbing the activities of microbial biomass away from the norm, with advantages coming from targeting of specific compounds providing insights into the wider fate of the isotopically-labelled substrate that

can never be achieved through bulk stable soil except by applying substrates at supra-normal rates. As we will show repeatedly below, the enhanced sensitivity of compound-specific approaches offers considerable, and in some cases hitherto unexplored, scope in both  $^{13}\text{C}$ - and  $^{15}\text{N}$ -SIP studies.

One final important point is that the most successful SIP experiments are predicated on the use of highly enriched stable isotopically-labelled substrates in ‘pulse-chase’ or continuous incubation experiments. Such experiments have grown in popularity since they circumvent the sensitivity limitations experienced when using natural abundance-labelled substrates, e.g.  $\text{C}_4$  plant litters or animal dung. While such substrates might have appeal on grounds of greatly reduced cost and availability in large amounts, except in some special circumstances (e.g. Dungait *et al.*, [2008, 2009]), their applications will be significantly more limited than when isotopically-enriched substrates are employed.

## Dynamic-SIP

We define here *dynamic-SIP* as those experiments which involve regular sampling over a time-course throughout the incubation period. We actually regard such experiments as fundamental in optimising experimental designs in order to: (i) establish optimal incubation times, (ii) ensure that equilibrium conditions have been reached, (iii) detect changes in microbial populations with time, (iv) detect cross-feeding between microbes in long-term incubations, and (v) provide numerical estimates for nutrient fluxes and pool sizes. Given the hundreds of reported SIP studies it is surprising that dynamic-SIP experiments are performed so infrequently. Table 1 lists examples of dynamic  $^{13}\text{C}$ -SIP experiment performed during the past decade.

**Table 1.** Examples of dynamic  $^{13}\text{C}$ -SIP studies of the soil microbial biomass arranged by substrate applied.

Substrate	Environment	Biomarkers	Observation	Reference
Chemically defined				
$^{13}\text{CH}_4$	Rice field/forest soils	PLFAs	Methanotrophs affected by N fertilisation	[Mohanty <i>et al.</i> , 2006]
$^{13}\text{CH}_4$	Upland grassland	PLFAs	New approach to estimating methanotroph population characteristics	[Maxfield <i>et al.</i> , 2006]
$^{13}\text{CH}_4$	Upland grassland	PLFAs	Methanotrophs affected by inorganic fertilisation	[Maxfield <i>et al.</i> , 2008]
$^{13}\text{CH}_4$	Volcanic soils	PLFAs	High populations of methanotrophs in andisols	[Maxfield <i>et al.</i> , 2009]
$^{13}\text{CH}_4$	Rothamsted Classical Experiment	PLFAs	Methanotrophs affected by inorganic fertilisation	[Maxfield <i>et al.</i> , 2011]
$^{13}\text{CH}_4$	Landfill cap soil	Various cellular biochemicals	New technique for elucidating total C dynamics (incorporation, turnover and decay) at the molecular level	[Maxfield <i>et al.</i> , 2012]
Carbon dioxide				
$^{13}\text{CO}_2$	Douglas-fir forest soil	PLFAs	Differential utilisation of substrates; also affected by plant inputs.	[Brant <i>et al.</i> , 2006]
$^{13}\text{CO}_2$	Desert soil	PLFAs	Fungal-to-bacterial PLFA-C increased under elevated $\text{CO}_2$	[Jin and Evans, 2010]

$^{13}\text{CO}_2$	Upland grassland	PLFAs, DNA, RNA	compared to ambient conditions Complex C dynamics and pathways of photosynthate entering soil	[Leake <i>et al.</i> , 2006]
$^{13}\text{CO}_2$	Boreal pine forest soil	PLFAs	Photosynthate found mainly in fungi and Collembola with long-term C allocation affected by N additions	[Hogberg <i>et al.</i> , 2010]
$^{13}\text{CO}_2$	Sandy loam agricultural soil	PLFAs	Plant species influences magnitude and distribution of plant C to soil microorganisms	[Ladygina and Hedland, 2010]
$^{13}\text{CO}_2$	Sandy peat agricultural soil	PLFAs, DNA, RNA	C flow of photosynthate to soil varies between GM plant and parental cultivar	[Hannula <i>et al.</i> , 2012]
$^{13}\text{CO}_2$	Paddy soil (Hydragric Anthrosol)	PLFAs	Differing microbial communities established by manipulating water status	[Yao <i>et al.</i> , 2012]
$^{13}\text{CO}_2$	Fore-dune soil	PLFAs, RNA	Elevated $\text{CO}_2$ induced changes in rhizosphere C flow and dynamics	[Dirgo <i>et al.</i> , 2013]
$^{13}\text{CO}_2$	Drained boreal organic peat soil	PLFAs	Flow of $^{13}\text{C}$ maximised at 3 days in fungi but after 1 year $^{13}\text{C}$ increased in bacteria	[Tavi <i>et al.</i> , 2013]
$^{13}\text{CO}_2$	Mountain meadow dystic cambisol	PLFAs	Shading produced no reduction $^{13}\text{C}$ in fungi and bacteria but increased residence time	[Bahn <i>et al.</i> , 2013]
$^{13}\text{CO}_2$	Sandy grassland soil	PLFAs	Faster turnover of C in mycorrhizal versus saprotrophic fungi versus bacteria	[Balasooriya <i>et al.</i> , 2014]
$^{13}\text{CO}_2$	Jena Biodiversity Experiment	PLFAs, RNA	C turnover is higher in RNA than PLFAs	[Malik <i>et al.</i> , 2015]
Plant litter				
$^{13}\text{C}$ -, $^{15}\text{N}$ -litter	Sand-clay growth medium	PLFAs	Transition from fungal to bacterial PLFAs as decomposition progressed	[Herman <i>et al.</i> , 2011]
$^{13}\text{C}$ -aspen litter	Forest soil	PLFAs	Chronic N deposition decreased $^{13}\text{C}$ mobilisation into microbial biomass	[Gan <i>et al.</i> , 2013]
$^{13}\text{C}$ -barley residues	Humid and semi-arid Cambisol and Haplic Kastanozem	PLFAs	Overall plant residue turnover slower under semi-arid conditions	[Helgason <i>et al.</i> , 2014]
$^{13}\text{C}$ -eucalyptus leaf litter	Remnant native woodland soil	PLFAs, DNA, RNA	Distinct microbial communities alter turnover and fate of OM in response to temperature	[Creamer <i>et al.</i> , 2015]
$^{13}\text{C}$ -wheat grains, leaves, roots	Conventionally tilled Luvisol	PLFAs	Double exponential model described PLFA synthesis-degradation kinetics	[Bai <i>et al.</i> , 2016]
Microbial cells				
$^{13}\text{C}$ - <i>E.coli</i>	Agricultural reference soil	PLFAs, DNA	Biomass C retained in soil in soil food web and non-living SOM	[Kindler <i>et al.</i> , 2006]
$^{13}\text{C}$ - <i>E.coli</i>	Agricultural reference soil	PLFAs, DNA	Biomass C recycled down to 10% in living organisms with 33% stabilised in non-living SOM pool	[Kindler <i>et al.</i> , 2009]
$^{13}\text{C}$ -methanotrophs	Rice field soil	PLFAs	Eukaryotes, probably protozoa, preferentially graze on methanotrophs	[Murase <i>et al.</i> , 2011]

As can be seen from Table 1 these studies include both laboratory and field experiments and a wide range of  $^{13}\text{C}$ -labelled substrates. Dynamic-SIP approaches can be divided into two broad categories, namely those applying: (i) complex substrates, such as  $^{13}\text{C}$ -labelled plant litter [Williams *et al.*, 2006; Moore-Kucera and Dick, 2008; Herman *et al.*, 2012; Gan *et al.*, 2013; Helgason *et al.*, 2014; Creamer *et al.*, 2015; Bai *et al.*, 2016] or  $^{13}\text{C}$ -labelled microbial cultures [Kindler *et al.*, 2006, 2009; Murase *et al.*, 2011], or (ii) pure  $^{13}\text{C}$ -labelled compounds [Brant *et al.*, 2006; Bartling *et al.*, 2009; Mellendorf *et al.*, 2010; Jakobs-Schonwandt *et al.*, 2010; Dungait *et al.*, 2011; Churchland *et al.*, 2012; Apostel *et*

*al.*, 2013; Lemanski and Scheu, 2014].  $^{13}\text{CO}_2$  pulse-chase experiments are amongst the most widely applied, designed to deliver  $^{13}\text{C}$ -labelled root exudate derived from newly formed  $^{13}\text{C}$ -photosynthate to explore C allocation from plants to the below-ground biomass [Leake *et al.*, 2006; Jin and Evans, 2010; Hogberg *et al.*, 2010; Ladygina and Hedland, 2010; Hannula *et al.*, 2012; Yao *et al.*, 2012; Dirgo *et al.*, 2013; Tavi *et al.*, 2013; Bahn *et al.*, 2013; Dias *et al.*, 2013; Balasooriya *et al.*, 2014; Malik *et al.*, 2015]. In both categories  $^{13}\text{C}$ -PLFAs are the most commonly investigated biomarkers with time-courses varying widely (days to years) to provide temporal and quantitative insights into the processing of  $^{13}\text{C}$ -labelled substrates by the soil microbial community, often in response to changes in particular environmental variables e.g. [Creamer *et al.*, 2015]. A small number studies have built in DNA and RNA-SIP investigations to provide deeper taxonomic assessments e.g. [Leake *et al.*, 2006].

### **Dynamic $^{13}\text{CH}_4$ stable isotope probing**

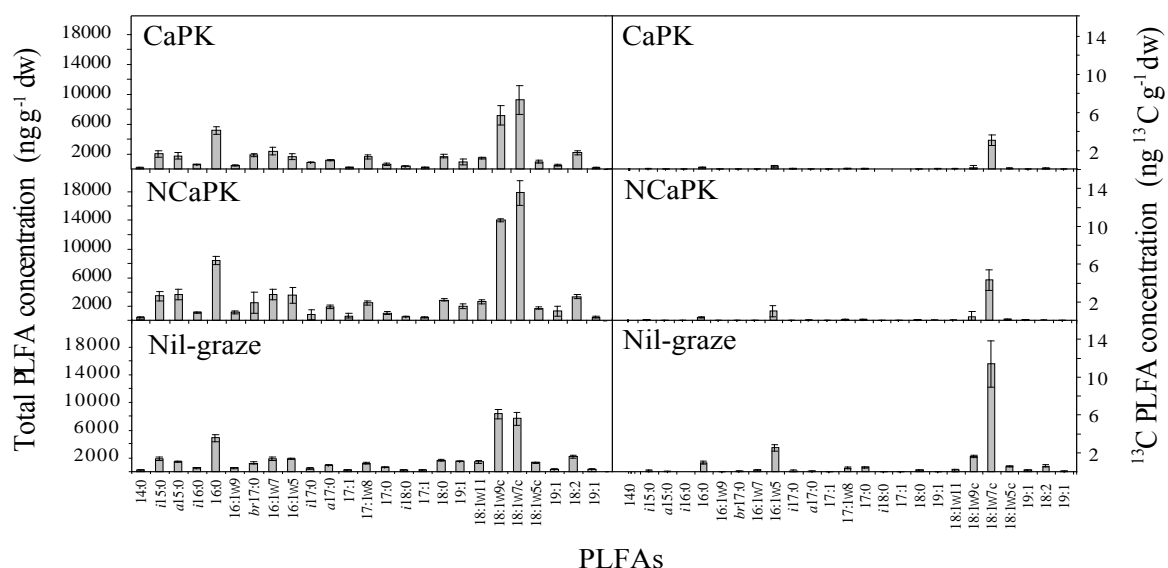
Dynamic  $^{13}\text{CH}_4$  stable isotope probing has been extensively employed in studies of methanotrophic communities in soils (Table 1). The approach has proven especially effective in the case of the unculturable high-affinity methanotrophic bacteria where an active methanotrophic population assimilating  $^{13}\text{CH}_4$  can be confirmed by  $^{13}\text{C}$ -enriched PLFAs detected by GC-C-IRMS. Indeed, as noted above, one of the first applications of  $^{13}\text{C}$ -PLFA to soil microbes was in the study of methanotrophs [Bull *et al.*, 2000]. We have extended this technique to characterise methanotrophic bacteria based on a combination of  $^{13}\text{C}$ -labelled hopanoids and PLFAs [Crossman *et al.*, 2001, 2005], recently incorporating this approach into a new type of  $^{13}\text{CH}_4$  dynamic- SIP experiment, termed stable isotope switching (SIS), which we will discuss in detail below [Maxfield *et al.*, 2012].

A major development of this approach has been our implementation of  $^{13}\text{CH}_4$  time-series  $^{13}\text{C}$ -PLFA labelling which facilitated, for the first time, enumeration of high affinity methanotroph populations [Maxfield *et al.*, 2006]. This approach involves performing laboratory incubations of soils at ambient  $\text{CH}_4$  concentrations using synthetic air containing 2 ppmv of  $^{13}\text{CH}_4$ . Flow through chambers are employed to maintain a stable  $^{13}\text{CH}_4$  concentration throughout the 11-week incubations (see Maxfield and Evershed [2011] for chamber design). When using this approach soils are sampled and analysed at weekly intervals by GC, GC-MS, and GC-C-IRMS to identify and quantify individual PLFAs and trace the incorporation of  $^{13}\text{C}$ -label into the microbial biomass. Incorporation of the  $^{13}\text{C}$ -label is generally seen throughout the experiment, with the rate of incorporation decreasing in the later stages (9 to 10 weeks) as the methanotroph population becomes fully  $^{13}\text{C}$ -labelled. The  $\delta^{13}\text{C}$  values of individual PLFAs showed that  $^{13}\text{C}$  was incorporated into different components selectively and at various rates, reflecting the diversity of PLFA sources. Quantitative assessments of  $^{13}\text{C}$ -PLFAs have shown that high affinity methanotrophic populations are usually of constant structure throughout the experiment. In the original experiment, performed using soils from Bronydd Mawr (Wales), the



dominant  $^{13}\text{C}$ -labelled PLFA was 18:1 $\omega$ 7c, with 16:1 $\omega$ 5 present at lower abundance, suggesting the presence of novel type II methanotrophs. We used the concentration of PLFAs per bacterial cell determined by Frostegård and Bååth, [1996] to determine the biomass of  $\text{CH}_4$ -oxidizing bacteria; at optimum labelling this was estimated to be about  $7.2 \times 10^6$  cells  $\text{g}^{-1}$  of soil (dry weight), remarkably, only one ten thousandth of the total bacterial population! Cross-feeding or recycling of biomass C is a recognised phenomenon that may thwart some types of SIP investigation, resulting in a loss of specificity. However, due to the low concentration and high specificity of the  $^{13}\text{CH}_4$  labelled-substrate two factors supported this approach with high affinity methanotrophs: (i) recycling of  $^{13}\text{C}$ -label from the methanotrophic biomass is a slower process than initial  $^{13}\text{CH}_4$  incorporation, with only about 5 to 10% of  $^{13}\text{C}$ -PLFAs reflecting this process, and (ii) the relatively low cell numbers of high affinity methanotrophs compared to the total microbial biomass ( $10^6$  versus  $10^{10}$  cells, respectively) means that the any leakage of  $^{13}\text{C}$ -label into other microbial pools will effectively constitute a loss of label by dilution into the high background C pools, such that the  $^{13}\text{C}$ -PLFA profile of the high affinity methanotroph population will dominate. Thus,  $^{13}\text{C}$ -labelled PLFA distributions determined at any time point during  $^{13}\text{CH}_4$  incubation can be used for chemotaxonomic assessments, however, extended incubations are required to achieve maximum  $^{13}\text{C}$ -labelling for methanotrophic biomass determinations.

We have applied this technique in different environments to study variations in low and high affinity methanotrophic populations in relation to a range of environmental variables. For example, as shown in Figure 2, we have linked a reduction of  $\text{CH}_4$  sink capacity to inhibition of the high affinity methanotroph populations resulting from long-term inorganic fertiliser applications to upland grassland soils [Maxfield *et al.*, 2008]. While it is well known that agricultural practices, including tillage [Hutsch, 1998] and fertilizer addition [Steudler *et al.*, 1989; Mosier *et al.*, 1991; Adamsen and King, 1993; Castro *et al.*, 1995; Gullledge *et al.*, 1997; Gullledge and Schimel, 1998], inhibit  $\text{CH}_4$  oxidation in soils much debate surrounds the proposed mechanism(s).



**Figure 2.** Concentrations of the extracted total PLFAs compared with  $^{13}\text{C}$ -PLFA concentration following 17–18 weeks of incubation under 2 ppmv  $^{13}\text{CH}_4$  for Bronydd Mawr NCaPK, CaPK and Nil-graze soils. Error bars represent  $\pm 1$  SD.

Our results show that inhibition of high-affinity methanotrophic bacteria in the Bronydd Mawr long-term low-input fertilized soils is caused by a general fertilization-induced salt effect rather than a nitrogen-specific inhibitory mechanism. Addition of  $\text{NH}_4^+$  to soils has been reported to suppress  $\text{CH}_4$  uptake rates [Mosier *et al.*, 1991; Adamsen and King, 1993; Castro *et al.*, 1995; Gullledge *et al.*, 1997; Gullledge and Schimel, 1998]. Inhibition also has been observed in short-term studies [Gullledge *et al.*, 1997; Wang and Ineson, 2003; Crossman *et al.*, 2006] for a range of ions including  $\text{K}^+$ ,  $\text{Cl}^-$  and  $\text{SO}_4^{2-}$ , confirming that general salt effects rather than by-products of  $\text{NH}_4^+$  oxidation can inhibit  $\text{CH}_4$  oxidation. Reay and Nedwell [2004] observed a clear relationship between inhibition of  $\text{CH}_4$  oxidation and  $\text{NO}_3^-$  rather than  $\text{NH}_4^+$ . However, the mechanism of  $\text{CH}_4$  oxidation inhibition by  $\text{NO}_3^-$  rather than  $\text{NO}_3^-$  and  $\text{NH}_4^+$  remains unclear. Gullledge and Schimel [1998] demonstrated the importance of non-ammonical salts on  $\text{CH}_4$  oxidation by treating soils with  $\text{K}_2\text{SO}_4$ ,  $\text{Na}_2\text{SO}_4$ ,  $\text{KCl}$ ,  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{NH}_4\text{Cl}$ . Methane oxidation was inhibited to some extent by all the salts irrespective of  $\text{NH}_4^+$  levels, which were naturally low in the soils. It was suggested that inhibition was not a result of desorption of  $\text{NH}_4^+$  from the soils but rather a direct effect of  $\text{SO}_4^{2-}$  or  $\text{Cl}^-$  ions on  $\text{CH}_4$  oxidation.

With the exception of two recent laboratory studies, all previous investigations have used changes in  $\text{CH}_4$  oxidation rates to detect patterns of inhibition, thereby making it impossible until now to explain the underlying cause of inhibition. Our results confirm that loss of  $\text{CH}_4$  oxidation capacity is the result of a shift in microbial populations resulting in a significant decrease in the numbers of methanotrophic bacteria. Our results also confirmed why problems have been encountered in studying the mechanism(s) of fertilizer addition impacts on high-affinity methanotrophic bacteria. The low relative abundance ( $<0.04\%$ ) of methanotrophs in total microbial biomass precludes use of other culture independent microbiological methods, such as earlier attempts using gene probing [Reay *et al.*, 2001; Seghers *et al.*, 2003] and most probable number (MPN) techniques [Hütsch, 2001]. Frustratingly, the exact mechanism(s) underlying the loss of high affinity methanotrophic activity remains unknown but it is clear that the loss of  $\text{CH}_4$  sink capacity as judged by flux measurement and methanotroph population estimates can be as much as 80%.

Similar effects of fertiliser additions were originally observed through flux measurements on the arable soils of Rothamsted by Powlson and co-workers [Hütsch *et al.*, 1993; Willison *et al.*, 1995]. Our more recent work with dynamic  $^{13}\text{CH}_4$   $^{13}\text{C}$ -PLFA-SIP using flow-through chambers has confirmed the very small methanotroph populations in these heavily fertilised and extensively tilled soils [Maxfield *et al.*, 2011]. Overall, methanotrophic biomass was smaller in the Rothamsted soils compared with the other mineral soils studied to date, as indicated by poor  $^{13}\text{C}$  incorporation. High-affinity methanotrophs similar to known type II methanotrophs were most abundant in all of the soils

studied, except in two plots receiving farmyard manure (FYM and FYM + N). Manuring resulted in a shift to a population similar to known type I methanotrophs. Methanotrophic biomass was elevated in soils that had received the largest input of N fertilizer, though without detectable differences in CH<sub>4</sub> oxidation rates, indicating the potential mediation of atmospheric CH<sub>4</sub> oxidation by non-methanotrophs, which are most likely to be nitrifying bacteria.

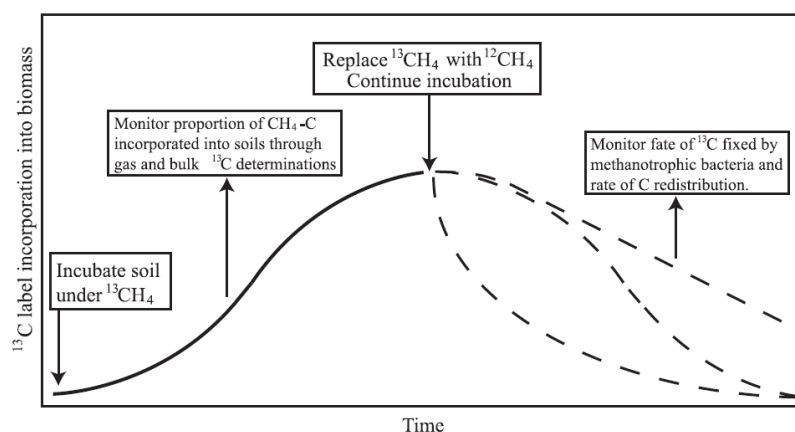
These results clearly demonstrate the impact that inorganic fertilizers have on the capacity of soil to function as a sink for tropospheric CH<sub>4</sub> and provide a strong link to changes in high-affinity methanotroph community structure. Placing these findings in the wider context of greenhouse gas emissions and mitigation strategies requires consideration of national and international inventories for CH<sub>4</sub> sources and sinks. Significantly, the UK has the highest anthropogenic CH<sub>4</sub> emissions in Europe after France [Bergamaschi *et al.*, 2005] with the largest source of CH<sub>4</sub> emissions in the UK being agriculture [NETCEN, 2003]. The lack of a detailed UK inventory of CH<sub>4</sub> sources and sinks precludes calculation of an accurate estimation of the proportion of the UK CH<sub>4</sub> sink inhibited by inorganic fertilizer (N + non-N). Our conservative estimate is that low-input fertilizer inhibition of high-affinity methanotrophic bacteria in the UK is as much as 5% of the EU-15 soil sink. Considering that the surface area of the UK (244 x 10<sup>3</sup> km<sup>2</sup>) is less than 10% of the total EU-15 surface area (3154 x 10<sup>3</sup> km<sup>2</sup>) then inhibition due to low-input fertilization may be > 50% of the potential UK soil CH<sub>4</sub> sink. Thus, the regional and indeed global impacts of inorganic fertilizer treatments on soil microbial diversity as an integral component of system C dynamics are worthy of continued detailed assessment.

Indeed, we have extended this work to other soil types. Andisols, soils derived from volcanic ash, which have the capacity to support high levels of atmospheric CH<sub>4</sub> oxidation [Singh *et al.*, 2007]. The unique properties of Andisols, specifically their low bulk density, high C content and high water-holding capacity, would provide a favourable environment for the development of methanotroph populations. The low bulk density in particular would promote gas diffusion, enhancing the supply of CH<sub>4</sub> and O<sub>2</sub> while maintaining a relatively high moisture content. Furthermore, high-affinity methanotrophs thrive in soils that have good substrate and moisture supplies. We characterized methanotrophic bacteria in Andisols *via* the dynamic <sup>13</sup>CH<sub>4</sub> SIP time-series <sup>13</sup>C-PLFA labelling approach discussed above. Three Andisols were incubated under 2 ppmv CH<sub>4</sub> for up to 18 weeks, enabling high-affinity methanotrophs to be selectively characterized and quantified. PLFA profiles from all soils were broadly similar, but the magnitude of the high-affinity methanotrophic populations determined through <sup>13</sup>C-PLFA-stable isotope probing displayed sizeable differences. Substantial incorporation of <sup>13</sup>C indicated very large high-affinity methanotrophic populations in two of the soils. Such high values are far in excess (10x) of those observed for the afore-mentioned mineral soils incubated under similar conditions [Maxfield *et al.*, 2008, 2011]. Two of the three Andisols studied also displayed high but variable CH<sub>4</sub> oxidation rates ranging from 0.03 to 1.58 nmol CH<sub>4</sub> g<sup>-1</sup> d.wt. h<sup>-1</sup>.

These findings suggest that Andisols may oxidize significant amounts of atmospheric CH<sub>4</sub> despite their low area of coverage globally. Andisols cover only *c.* 0.7% of the global land surface [Brady and Weil, 2002], which equates to a land area of *c.* 0.91 million km<sup>2</sup>. Total uptake of atmospheric CH<sub>4</sub> globally by Andisols may be greater than previously thought if methanotrophic activity is similar to the rates observed in the Hapludand and Ultic Fulvudand soils we investigated. The much lower levels of methanotrophic activity displayed a greater net CH<sub>4</sub> flux suggesting the possible role of low-affinity methanotrophs at the site. It should also be noted that Andisols are extensively cultivated globally, in particular, in Japan and Italy because of their intrinsically high C content and favourable physical properties. As discussed above it is well established that certain agricultural practices, for example, the application of inorganic N-fertilizers inhibit the activity of methanotrophic bacteria in mineral soils e.g. [Mosier *et al.*, 1991; Gullledge *et al.*, 1997; Boeckx *et al.*, 1998; Hütsch, 1998]. A similar relative degree of inhibition of methanotrophy in Andisols may represent a much greater loss of atmospheric CH<sub>4</sub> oxidation capacity.

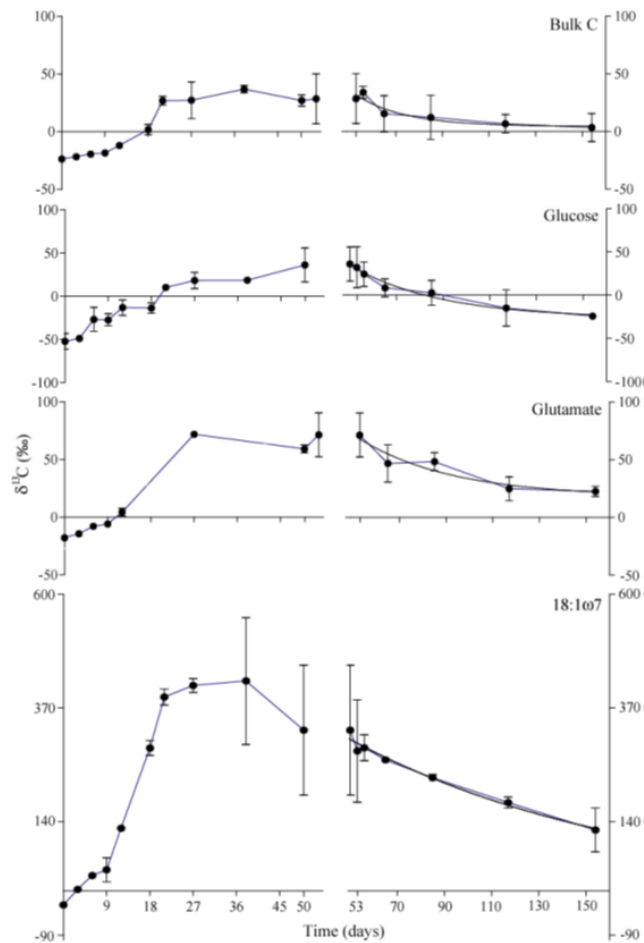
### Stable isotope switching (SIS)

Recent advances in stable isotope probing (SIP) have enabled direct linkage of microbial population structure and function, however investigations of the redistribution of <sup>13</sup>C-label in C pools other than PLFAs and DNA/RNA are rare. To address this we introduced a new SIP approach, *stable isotope switching* (SIS), which enables the simultaneous assessment of C uptake, turnover and decay, and the elucidation of soil food webs within complex soils or other environmental matrices [Maxfield *et al.*, 2012]. SIS utilises a stable isotope labelling approach whereby the <sup>13</sup>C-labelled substrate is switched part way through the incubation to a natural abundance substrate, which allows the reallocation of C following fixation by methanotrophs. Figure 3 shows a schematic of the experimental design and potential uptake and redistribution trajectories. Regular sampling throughout the experiment is a prerequisite of the approach.



**Figure 3.** Schematic representation of a stable isotope switching (SIS) experiment based on previously observed (solid line) and theoretical (dashed lines) curves indicating how the  $^{13}\text{C}$ -label from  $^{13}\text{CH}_4$  is incorporated by methanotrophs and turned-over within soil ecosystems. The timescale to achieve full labelling of the microbial population will vary depending on the labelled substrate, concentration, delivery method and the nature of the environmental matrix studied (modified from Maxfield *et al.*, [2012]).

The development work used a landfill cover soil from Odcombe (Somerset, UK) which previous work had shown contained a high abundance of low affinity methanotrophs [Crossman *et al.*, 2004]. During an incubation lasting 153 days C assimilation and dissimilation processes were monitored through bulk IRMS and GC-C-IRMS targeting a wide range of biomolecular components including: lipids, proteins and carbohydrates (Fig. 4). Carbon assimilation by primary consumers (methanotrophs) and sequential assimilation into secondary (Gram-negative and -positive bacteria) and tertiary consumers (Eukaryotes) was observed (the full experimental details for sample preparation and data analysis are given in Experimental Methods at the end of this chapter and in Maxfield *et al.*, [2012]). Mass balance calculations showed that up to 45% of the bacterial membrane lipid C was directly derived from  $\text{CH}_4$  and at the conclusion of the experiment *ca.* 50% of the bulk soil C derived directly from  $\text{CH}_4$  was retained within the soil. At the time this work was reported it appeared that this was the first estimate of soil organic C allocation from methanotrophy. The high concentration of C retained is comparable with levels observed in lakes that have high levels of benthic methanogenesis. SIS opens the way for a new generation of SIP studies aimed at elucidating total C dynamics (incorporation, turnover and decay) at the molecular level in a wide range of complex environmental and biological matrices.



**Figure 4.** Graphs showing the differential rates and extents of uptake of the  $^{13}\text{C}$ -label into the bulk soil C pool and different biochemical components of the microbial community of a landfill cap soil after 50 d of incubation with only 1 atm%  $^{13}\text{CH}_4$  (1%  $\text{CH}_4$  balance synthetic air) in a flow through chamber. The different time points derive from the analysis of replicate soils sampled at intervals throughout the experiment. The right hand side graphs show the decay of the label in the different pools after switching to  $^{12}\text{CH}_4$  on day 50. The data obtained clearly illustrate the new levels of C pool and kinetic data available through our new approach. 18:1 $\omega$ 7 is a PLFA known to be produced by methanotrophs hence the high rate and extent of  $^{13}\text{C}$ -label into this compound.

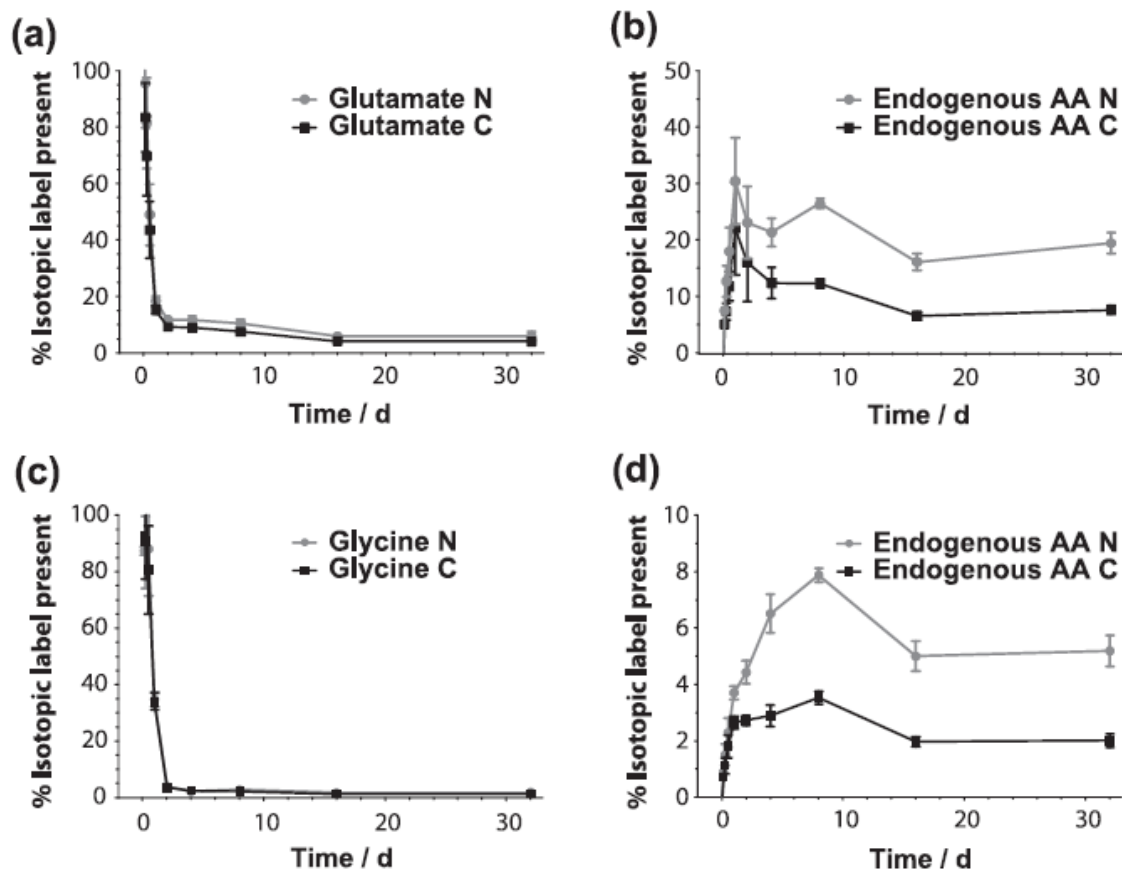
### Amino acid stable isotope probing (AA SIP)

As demonstrated in the SIS experiment described above and in the work by Derrien *et al.* [2007] summarised in Table 1, small molecule SIP using GC-C-IRMS has been extended beyond PLFAs to a wider range of biomarkers/analytes (e.g. sugars and AAs). As N-containing molecules, AAs also raise the possibility for  $^{15}\text{N}$ -SIP incubation experiments. We initially used combined  $^{13}\text{C}$  and  $^{15}\text{N}$ -SIP of AAs to trace the fates of N and C within metabolites central to soil organic N cycling [Knowles *et al.*, 2010] and then during work focusing on the transformations and fate of fertiliser-N compounds in soils, we further developed our conceptual use of the approach to provide a powerful tool to assess microbial assimilation and newly synthesised soil protein [Charteris *et al.* in press].

Knowles *et al.* [2010] traced the fate of  $^{13}\text{C}$  and  $^{15}\text{N}$  from dual-labelled U- $^{13}\text{C}$ ,  $^{15}\text{N}$ -glutamate and U- $^{13}\text{C}$ ,  $^{15}\text{N}$ -glycine into *de novo* biosynthesised AAs in laboratory time-course experiments. Glutamate

was selected as a substrate because of its centrality to cellular AA biochemistry, while glycine was chosen because of its more peripheral position in AA biosynthetic pathways. The AAs (2 µg) were applied to a grassland soil in laboratory mesocosms (10 g) and the fate of the  $^{13}\text{C}$  and  $^{15}\text{N}$  followed by sampling at  $t = 3, 6, 12, 24, 48, 96, 192, 384$  and 768 hours. In a preliminary experiment sampling was begun after 1 day but it was clear from the analyses that the initial phase of processing had been missed so the experiment was repeated with an earlier sampling time being included to capture the full trajectory of assimilation. Hydrolysable soil AAs were extracted, derivatised and their  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values determined using GC-C-IRMS as described in the Experimental Methods section at the end of the chapter.

$^{15}\text{N}$ - and  $^{13}\text{C}$ -stable isotopic analyses of soil AAs demonstrated that both  $^{15}\text{N}$  and  $^{13}\text{C}$  from glutamate and glycine were incorporated into other, endogenous AAs through *de novo* biosynthesis using N and C from these labelled substrates. The total proportions of  $^{15}\text{N}$  and  $^{13}\text{C}$  used in the *de novo* biosynthesis of endogenous AAs and remaining within glutamate and glycine were compared. As anticipated it was found that glutamate and glycine functioned differently as biochemical substrates in the biosynthesis of new AAs, with clear differences existing in the degree of N and C utilisation. The higher peak values of 30% glutamate N and 22% glutamate C being used in the synthesis of other AAs versus 8% glycine N and 2.8% glycine C, reflects their different positions in AA biosynthetic pathways. The total amount of label incorporated into other AAs began to decrease after  $t = 8$  d probably as a result of N and C mineralisation and their use in the biosynthesis of new cellular biochemicals. By applying linear and non-linear regressions, several important parameters were derived, namely rate constants, magnitudes of fluxes and measures of biosynthetic proximity, which describe the rate and magnitude of N and C flux through primary metabolic processes. The significant differences in N and C processing demonstrate a decoupling of the N and C cycles at the molecular level (Fig. 5), i.e. after 32 days the magnitude of N flux into newly biosynthesised AAs was double that of C from both substrates. We anticipate that the parameters derived will have potential for use in developing detailed models of soil organic N and C processing at the molecular level [Knowles *et al.*, 2010].



**Figure 5.** Variation (%) substrate label within the applied AA and endogenous soil AAs over time. Panels show the % of glutamate-derived N and C over time within (a) soil glutamate, and (b) endogenous soil AAs in U-<sup>13</sup>C, <sup>15</sup>N-glutamate amended soils; and the % of glycine-derived N and C over time within (c) soil glycine and (d) endogenous soil AAs in U-<sup>13</sup>C, <sup>15</sup>N-glycine-amended soils. Note different scales on y-axis; error bars represent  $\pm$  SEM ( $n = 3$ ).

As noted above, application of this approach to investigations of the transformations and fate of fertiliser-N compounds in grassland soils prompted its development into a more widely applicable conceptual technique to trace the fate of applied N in soils and indeed other complex ecosystems [Charteris *et al.* in press]. Compound-specific AA <sup>15</sup>N-SIP using GC-C-IRMS offers a number of insights, inaccessible via existing techniques, providing:

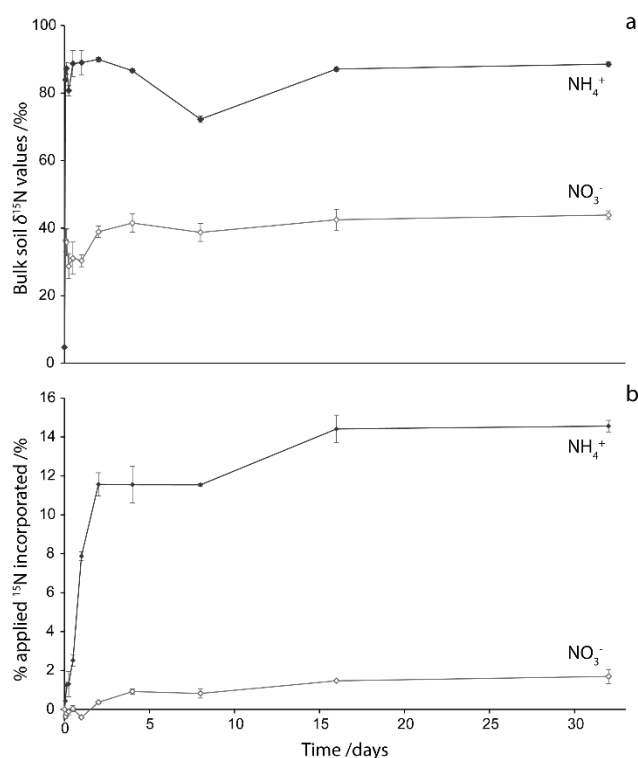
- (i) A sensitive and relatively selective means of assessing microbial assimilation of <sup>15</sup>N-labelled substrates/amendments applied at environmentally relevant concentrations and appropriately low <sup>15</sup>N enrichments to minimise perturbations and <sup>15</sup>N discrimination/isotopic fractionation, respectively (bulk N isotope analysis cannot provide such insights and GC/MS <sup>15</sup>N-SIP studies require highly <sup>15</sup>N-labelled substrates for sufficient product AA <sup>15</sup>N enrichment, making such studies more susceptible to <sup>15</sup>N discrimination/isotopic fractionation effects and are much more expensive).



- (ii) Valuable insights into microbial biochemical assimilation pathways can be gained and differences are readily revealed in the microbial processing of N-containing substrates of differing chemical/biochemical natures.
- (iii) Detailed quantitative insights can be gained into the dynamics of N cycling from an applied substrate through the soil protein pool.
- (iv) Estimates are provided for newly synthesized soil protein, which are inaccessible based on currently available methods.

Our compound-specific AA  $^{15}\text{N}$ -SIP method is particularly valuable in determining the fate of N-fertilisers in relation to the soil organic N (mainly biomass) pool as organic N greatly exceeds inorganic N in soils and few other techniques are able to provide direct quantitative estimates of this partitioning (perhaps with the exception of microbial biomass N extraction; [Charteris *et al.* in press]). Accordingly, laboratory time-course incubation experiments were undertaken with the common inorganic N fertiliser compounds  $^{15}\text{NH}_4^+$  and  $^{15}\text{NO}_3^-$  and the high sensitivity and selectivity GC-C-IRMS was again exploited to determine the assimilation of the applied  $^{15}\text{N}$ -labelled substrate by the soil microbial biomass and provide estimates of newly synthesized soil protein (see protocols at the end of the chapter). As with the glutamate and glycine incubations summarised above, the assimilation pathways of  $^{15}\text{NH}_4^+$  and  $^{15}\text{NO}_3^-$  were revealed via patterns in AA  $\delta^{15}\text{N}$  values with time, reflecting known biosynthetic pathways (e.g. ammonium uptake occurs first via glutamate). Comparing these data to those for organic substrates such as glutamate, clear differences can be seen in the rates and fluxes of the applied N substrates into the soil protein pool (glutamate > ammonium > glycine > nitrate). This work constitutes a significant advance as the cycling of agriculturally relevant N additions is undetectable via bulk soil N content and  $\delta^{15}\text{N}$  measurements and AA concentrations (Figure 6).

Although it does not include a time-course, the work of Redmile-Gordon *et al.* [2015] provides an example of how this conceptual compound-specific  $^{15}\text{N}$  SIP approach might be extended or refined by considering different soil protein fractions. Finally, the method could potentially be adapted to investigate N cycling into other N-containing biochemical pools, e.g. amino sugars, as appropriate extraction, derivatisation and GC-C-IRMS protocols are developed.



**Figure 6.** Bulk  $\delta^{15}\text{N}$  values of whole soil (a) are insensitive to changes in N cycling during the incubation of  $^{15}\text{N}$ -labelled fertilisers at field application rates.  $^{15}\text{N}$  AA SIP allows the percentage of applied  $^{15}[\text{NH}_4]^+$  and  $^{15}[\text{NO}_3]^-$  incorporated (b) into the total hydrolysable AA pool or soil protein pool to be determined. Error bars are  $\pm$  SE ( $n=3$ ). Calculations for  $^{15}[\text{NH}_4]^+$  and  $^{15}[\text{NO}_3]^-$  are straightforward summations of the percentage of the applied  $^{15}\text{N}$  incorporated into each AA, indicating considerable difference in the use of the applied  $^{15}\text{N}$  in *de novo* AA biosynthesis.

## Looking to the future

*Dynamic-SIP* is a proven approach in soil research with applications involving a wide range of cellular biochemical products. The survey of recent published examples show that there has been a focus on DNA/RNA- and PLFA-SIP, mainly as taxonomic tools, to trace the fate of  $^{13}\text{C}$ -label from applied substrates in the soil microbial biomass. In some cases the magnitude of  $^{13}\text{C}$ -labelling is used as an indication of the relative importance and different taxonomic groups receiving the label, both spatially and temporally (e.g. within the rhizosphere). From our preliminary work with the SIS technique, however, it is clear that there are additional benefits that accrue, when considering this methodology and its variants, beyond its use just as a taxonomic technique. We showed in our SIS studies that the stable isotope label from applied substrates can be traced into the full range of cellular biochemicals produced by soil biota. This opens up the SIP technique as a highly selective and sensitive technique for investigating the cycling of a wide range of soil natural substrates as well as agricultural inputs and amendments. Where dynamic-SIP has been used to determine the rates of uptake and turnovers of different biochemical pools it is clear that these can vary widely even within a compound class, e.g. AAs, as a result of the specific demands of different organisms and the

associated biochemical pathways. Such information has the potential to feed into a new generation of molecular-based SOM cycling models.

The emergence of SIP at the turn of the millennium was in large part driven by a combination of technological advances, notably the commercial availability of GC-C-IRMS, which enabled compound-specific stable C isotope analyses to be performed on soil derived PLFAs. As we have shown, a wide range of other cellular biochemical can be made amenable to GC-C-IRMS using the appropriate protocols, e.g. hydrolysis and derivatisation for biopolymers, such as proteins and carbohydrates. There is also scope for using high temperature-GC-C-IRMS, employing short columns with thin stationary phase coatings to determine stable isotope compositions of high molecular weight apolar lipids. However, new opportunities also exist to investigate the isotopic composition of polar and high molecular weight compounds using high performance liquid chromatography-IRMS or direct infusion IRMS. This instrument uses a solution rather gas phase oxidation to generate CO<sub>2</sub> from organic compounds for C isotope analysis and thereby offers as yet untapped potential for incorporating into soil SIP studies. Such an instrument would have value in determining  $\delta^{13}\text{C}$  values of genomic material isolated from density centrifugation gradients.

Additionally, numerous untapped opportunities exist to extend the applications of SIP with GC-C-IRMS to study other isotope systems, including  $^{15}\text{N}/^{14}\text{N}$ ,  $^2\text{H}/^1\text{H}$ ,  $^{18}\text{O}/^{16}\text{O}$ . We have shown, through our work above, the advantages of using compound-specific  $^{15}\text{N}$ - and  $^{15}\text{N},^{13}\text{C}$ -AA SIP to track the fate of nitrogenous substrates into newly biosynthesised biomass protein. The sensitivity and selectivity of GC-C-IRMS will enable a new generation of naturalistic experiments, to probe the functional behaviour of soil microbial biomass, to be performed. Such studies have hitherto been impossible to conduct using conventional GC-MS or bulk isotope approaches.

Finally, we cannot over emphasise that *dynamic*-SIP should be the norm in experimental design in this area. SIP experiments performed without regular sampling over a proper time-course cannot reveal the true functional dynamics of the soil microbial mass with respect a given substrate. Without this information we will never be able to properly predict the fate of substrates or biomass response to a given set of environmental variables. We accept that such experiments are considerably more demanding in terms of infrastructure resource and manpower, but not performing *dynamic*-SIP experiments will result in potentially vital aspects of soil biomass behaviour remaining undetected.

## **Acknowledgements**

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<http://www.lmsf.co.uk>). The UK Biochemistry and Biotechnology Research Council is also thanked for a PhD studentship (TDJK).

## Experimental Methods

### Dynamic $^{13}\text{CH}_4$ -SIP

This methodology follows that of Maxfield *et al.* [2006]. Soil from a depth of 5 cm was selected for the  $^{13}\text{CH}_4$  incubation due to its high  $\text{CH}_4$  oxidation rate ( $0.04 \text{ nM CH}_4 \text{ g}^{-1} \text{ dw h}^{-1}$ ). Soil samples for  $^{13}\text{CH}_4$  incubation were weighed (10 g) into 5 cm diameter petri dishes and placed into an incubation chamber (63 L; Perspex®) sealed at the base via immersion in a water-filled tray to prevent gas leakage. The water in the base of the chamber maintained atmospheric moisture saturation and prevented samples from drying. Double-distilled water (DDW) was added periodically (*ca.* 2 week intervals) to the soils during incubation to maintain moisture levels.  $^{13}\text{CH}_4$  (2 ppmv) pre-mixed in synthetic air was passed through the chamber at a rate of  $44 \text{ ml min}^{-1}$  in order to renew the headspace every 24 h. Each soil sample was incubated in triplicate with incubation times ranging from 2 to 11 weeks. A second, identical set of 'control' soils were incubated in a separate building under ambient  $\text{CH}_4$  (2 ppmv) levels. All incubations were carried out in temperature-controlled rooms at  $20^\circ\text{C}$ . Throughout the experiment selected soil samples were removed for isotope analysis and headspace was monitored by GC analysis to verify that  $\text{CH}_4$  oxidation activity was being maintained.

### SIS $\text{CH}_4$ incubation

SIS is the method developed by Maxfield *et al.* [2012]. Soils containing low affinity methanotrophs were incubated in the same flow through incubation chamber described above. Synthetic air premixed with  $\text{CH}_4$  (1.33%) was constantly flowed through the chamber to maintain a steady  $\text{CH}_4$  mixing ratio close to that observed at the sampling site. For the initial 50 days of the incubation 1% of the  $\text{CH}_4$  was  $^{13}\text{CH}_4$  (mixed from  $> 99\%$   $^{13}\text{C}$ ; CK Gas Products Ltd, Hook, UK; equivalent to 133 ppmv  $^{13}\text{CH}_4$ ). Following 50 days of the incubation the  $^{13}\text{CH}_4$  was switched and pre-mixed natural abundance  $\text{CH}_4$  (13300 ppmv) was flowed through the chamber. The flow rate through the chamber was maintained at  $44 \text{ mL min}^{-1}$  throughout the entire incubation period in order to flush the entire headspace every 24 h. Soil samples were removed, in triplicate, at regular intervals throughout the incubation ( $t = 0, 3, 6, 9, 12, 18, 21, 27, 38, 50, 53, 56, 65, 85, 117, 154$  days) and initial soil moisture contents were maintained through the regular addition of DDW to each sample (determined gravimetrically). All of the soil samples were stored at  $-20^\circ\text{C}$  until required for analysis. Soil samples were freeze dried and ground prior to extraction and analysed for PLFAs, total AAs, total monosaccharides glycolipids (GLFAs), free fatty acids (FFAs), hopanoids, sterols, *n*-alkanes and *n*-alkanols, as described below.

### $^{15}\text{N}$ - and dual $^{13}\text{C}$ , $^{15}\text{N}$ -AA SIP

The soil was air-dried sufficiently to allow sieving (2 mm) without smearing it, which would result in destruction of the microstructure, and adjusted to 50% water holding capacity (WHC) by addition of DDW through an atomizer. Incubation experiments were carried out in glass incubation tubes (10 cm x 2 cm i.d.), to which 10.0 g soil (6.06 g dry wt. equivalent) were added and gently compacted to a density of  $\text{ca. } 1.1 \text{ g cm}^{-3}$  (field density) by tapping the tubes on a solid surface. Incubations were carried out under aerobic conditions and soils were maintained at 50 % WHC. A study by Jones and Shannon [1999] showed that sieving soil reduced AA mineralisation rate for the first 24 h, but subsequent incubation (up to 40 d) revealed no difference in AA mineralisation rate. To ensure this period was exceeded, a pre-incubation period of 4 d was employed before soils received either  $^{15}\text{N}$ -labelled ammonium chloride ( $^{15}\text{NH}_4\text{Cl}$ , 10 atom %, 400  $\mu\text{g}$  in 200  $\mu\text{L}$  DDW; Sigma-Aldrich, St. Louis, USA),  $^{15}\text{N}$ -labelled potassium nitrate ( $\text{K}^{15}\text{NO}_3$ , 10 atom %, 400  $\mu\text{g}$  in 200  $\mu\text{L}$  DDW; Sigma-Aldrich, St. Louis, USA),  $^{15}\text{N}$ ,  $^{13}\text{C}$ -labelled glutamic acid and glycine ( $^{15}\text{N}$ ,  $^{13}\text{C}$ -Glu, 98 atom %, 2 mg in 200  $\mu\text{L}$  0.1 M hydrochloric acid; HCl;  $^{15}\text{N}$ ,  $^{13}\text{C}$ -Glu from Spectra Stable Isotopes, Columbia, USA and HCl was reagent grade from Fisher Scientific, Loughborough, UK) or for the control samples, DDW (200  $\mu\text{L}$ ). Substrates were introduced by injection and the needle was drawn up through the soil as the plunger was depressed in order to achieve a good distribution. The incubation experiments were

halted by immersion in liquid nitrogen (N<sub>2</sub>) to instantly cease all biological activity after periods of 1.5, 3, 6 and 12 h and 1, 2, 4, 8, 16 and 32 d in the dark and stored at -20 °C until freeze-drying. All incubations were carried out in triplicate so there were three tubes for each time point of each treatment (see also Knowles *et al.*, [2010]). The 10 atom % <sup>15</sup>N enrichment of <sup>15</sup>[NH<sub>4</sub>]<sup>+</sup> and <sup>15</sup>[NO<sub>3</sub>]<sup>-</sup> was chosen based on research highlighting changes in <sup>15</sup>N discrimination and isotopic fractionation in biological mechanisms at very high enrichments [Mathieu *et al.*, 2007 ; Tang and Maggi, 2012]. Nitrogen-15 enrichments of 10 atom % were considered low enough for these effects to be negligible. The <sup>15</sup>N-Glu incubation experiments were carried out earlier, hence the undesirably high <sup>15</sup>N enrichment of the applied Glu.

### **PLFA analysis**

Following incubation all soil samples were freeze-dried, homogenized by grinding and extracted using a modified Bligh-Dyer monophasic solvent system [Maxfield *et al.*, 2006]. Briefly, soil (2 g<sub>dw</sub>) was extracted using Bligh-Dyer solvent containing buffered water (0.05 M KH<sub>2</sub>PO<sub>4</sub>; pH 7.2)/chloroform/methanol in a ratio of 4:5:10 v/v/v. After addition of solvent (3 mL), the mixture was sonicated (15 min) and centrifuged (3200 rpm, 5 min). The supernatant was decanted and the residue extracted as above (×3). The extracts were combined and evaporated under N<sub>2</sub> to yield the total lipid extract (TLE). The TLE was separated into three fractions based on polarity using a 3 mL STRATA aminopropyl-bonded silica cartridge (Phenomenex). Fractions were eluted sequentially with 2:1 v/v dichloromethane/isopropanol; (8 mL; yielding neutrals), 2% acetic acid in diethyl ether (12 mL; free fatty acids) and methanol (8 mL; polar fraction including phospholipids). The PLFA components of the polar fraction were released by saponification (1 mL 0.5 M methanolic NaOH, 1 h, 80°C) and an internal standard added (10 µL of 0.1 mg mL<sup>-1</sup> solution of C<sub>19</sub> *n*-alkane in hexane). The PLFAs were then methylated using a 14% v/v BF<sub>3</sub>-methanol solution (100 µL; Aldrich, UK, 1 h, 80°C) and extracted with chloroform (3 x 2 mL). The phospholipid ester-linked fatty acid methyl esters (PLFAMES) were dissolved in hexane for analysis by GC, GC-MS and GC-C-IRMS (see below).

Dimethyl disulphide (DMDS) derivatives of mono-unsaturated fatty acids were prepared to facilitate determination of double-bond positions and geometries in monounsaturated fatty acids. A 100 µL aliquot of the methyl esters in dichloromethane was added to 0.25 M I<sub>2</sub>/diethyl ether (100 µL) and dimethyl disulphide (1 mL) and then heated (60°C, 24 h in the dark). Excess I<sub>2</sub> was removed by addition of aqueous Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (5%, 2 mL) and the DMDS derivatives extracted with hexane (2 x 2 mL) for GC-MS analysis (see below and Maxfield and Evershed [2011] for mass spectral interpretations).

### **Lipid extraction and fractionation**

Lipid class separation was achieved using a modified silicic acid fractionation protocol [Dickson *et al.*, 2009] to yield three fractions; simple lipids, glycolipids and phospholipids. Simple lipids were further fractionated using silicic acid columns into four fractions; hydrocarbons (eluted with hexane, 4 mL), ketones and wax esters (dichloromethane (DCM), 6 mL), alcohols (DCM/methanol (MeOH), 1:1 v/v, 4 mL), and polar poly-functionalised compounds (MeOH, 4 mL). Alcohols and hydrocarbons were subjected to a further urea adduction procedure to separate cyclic from acyclic components [Nwadinigwe and Nwobodo, 1994].

### **Trimethylsilylation**

The PLFA, GLFA and FFA fractions were methylated as above. As with standard GC analysis, all neutral polar lipids were derivatised with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) prior to analysis by GC, GC-MS and GC-C-IRMS [Evershed, 1994].

### **Extraction and derivatisation of carbohydrates**

The method of Blakeney *et al.* [1983], modified by Docherty *et al.* [2001], was employed to prepare alditol acetate derivatives of total monosaccharides. Soils were hydrolysed with H<sub>2</sub>SO<sub>4</sub> and the released monosaccharides reduced with NaBH<sub>4</sub> to their corresponding alditols; excess NaBH<sub>4</sub> was removed by the addition of glacial acetic acid. The alditols were acetylated by reaction with *N*-methylimidazole and acetic anhydride. A standard mix of rhamnose, fucose, galactose, mannose, xylose, arabinose glucose, inositol and pentaerythritol was prepared according to Docherty *et al.*

[2001]. DDW (400  $\mu\text{L}$ ) was added to the dry monosaccharide standards and the solutions derivatised as above. A 20  $\mu\text{g } \mu\text{L}^{-1}$  internal standard (I.S.) was made up by dissolving 0.04 g pentaerythritol in 2 mL 1 M  $\text{NH}_3$  solution. Corrections for added derivative C and propagation of errors followed Docherty *et al.* [2001].

### **Extraction, isolation and derivatisation of hydrolysable amino acids**

Finely ground, freeze-dried incubation soil samples (100 mg) were weighed into culture tubes and 100  $\mu\text{L}$  of norleucine (Nle; 400  $\mu\text{g mL}^{-1}$  in 0.1 M HCl; Sigma-Aldrich, St. Louis, USA) was added as an internal standard. Hydrolysis with 5 mL 6 M HCl was carried out at 100 °C for 24 h under an atmosphere of  $\text{N}_2$ . Acid hydrolysis extracts both free and proteinaceous AAs as well as catalysing the breakdown of living microbial biomass [Roberts and Jones, 2008]. The relatively harsh conditions are necessary for the cleavage of peptide bonds between hydrophobic residues (e.g. isoleucine; Ile, leucine; Leu and valine; Val), but also result in the deamination of asparagines; Asn) to aspartate (Asp) and glutamine (Gln) to Glu and the complete destruction of cysteine (Cys) and tryptophan (Trp; [Fountoulakis and Lahm 1998; Roberts and Jones, 2008]). The technique may also partially destroy serine (Ser; *ca.* 10 % loss), threonine (Thr; *ca.* 5 % loss) and tyrosine (Tyr; loss depends on level of trace impurities in hydrolysis agent [Roberts and Jones, 2008] and has the potential to hydrolyse AA chains from non-proteinaceous sources, such as peptidoglycan, resulting in an overestimation of some AAs, mostly alanine (Ala), Glu, lysine (Lys) and glycine (Gly; [Roberts and Jones, 2008]). The technique is, however, considered the most reliable method for determining the total protein content of soils [Roberts and Jones, 2008] and as such, we equate total hydrolysable AA concentrations to the size of the soil protein pool. The hydrolysis is performed under  $\text{N}_2$  as the presence of oxygen ( $\text{O}_2$ ) can induce the thermal breakdown of hydroxyl- and sulphur-containing AAs (e.g. methionine; Met, Ser, Thr and Tyr; [Roberts and Jones, 2008]). Hydrolysates were collected by centrifugation, dried under a stream of  $\text{N}_2$  at 60 °C and stored at 20 °C under 1 mL 0.1 M HCl. AAs were isolated from hydrolysates by cation exchange column chromatography using acidified Dowex 50WX8 200-400 mesh ion exchange resin (Acros Organics, New Jersey, USA; Metges *et al.*, [1997]). This was followed by conversion to their *N*-acetyl, *O*-isopropyl derivatives for analysis [Corr *et al.*, 2007; Knowles *et al.*, 2010]. Derivatising agents were supplied by Sigma-Aldrich (Steinheim, Germany): acetyl chloride was puriss. p.a. grade; trimethylamine had  $\geq 99.50$  % purity; and acetic anhydride was ReagentPlus® grade. All solvents were of HPLC grade and were supplied by Rathburn Chemicals Ltd. (Walkerburn, Scotland). DDW was produced using a Bibby Aquatron still. Where not applied to living soil, DDW was extracted with dichloromethane prior to use in order to remove dissolved organic compounds.

### **Instrumental analyses**

**GC** analyses were performed using a Hewlett-Packard Series 5890 Series II gas chromatograph (Agilent Technologies UK Ltd., Edinburgh, UK) with a flame ionisation detector (FID) and  $\text{H}_2$  carrier gas (10 psi). Non-polar fractions were analysed using a Chrompack CPSil-5CB (50 m  $\times$  0.32 mm i.d.  $\times$  0.12  $\mu\text{m}$  film thickness). The temperature programme was 50°C to 200°C at 10°C  $\text{min}^{-1}$ , to 300°C at 3°C  $\text{min}^{-1}$  (held for 20 min). Polar compounds were analysed using a Varian VF23ms (Varian BV, Middelburg, The Netherlands) 50% cyanopropyl equivalent fused-silica column (60 m  $\times$  0.32 mm i.d.  $\times$  0.25  $\mu\text{m}$  film thickness). The temperature programme for fatty acid derivatives was 50°C (2 min) to 100°C at 15°C  $\text{min}^{-1}$ , to 240°C at 4°C  $\text{min}^{-1}$  (held for 20 min). The temperature programme for monosaccharides was 50°C (1 min) to 200°C at 20°C  $\text{min}^{-1}$ , to 230°C at 4°C  $\text{min}^{-1}$ , (held for 22 min). The temperature programme for amino acids was 40°C (1 min) to 120°C at 15°C  $\text{min}^{-1}$ , to 190°C at 3°C  $\text{min}^{-1}$ , to 250°C at 5°C  $\text{min}^{-1}$  (held for 20 min).

**GC-MS** analyses were performed using a Thermo Finnigan Trace GC-MS (Thermo Fisher Scientific, Hemel Hempstead, UK). All the GC conditions were the same with the exception of helium being used as carrier gas. The interface was held at the maximum oven temperature, the ion source was held at 200°C and the quadrupole mass analyser operated in EI mode, scanning over the range  $m/z$  50-650 at 1.7 scans  $\text{s}^{-1}$ . The emission current was maintained at 300  $\mu\text{A}$  and electron energy was 70 eV. The

data were acquired and analysed using the XCalibur software Version 1.2 (ThermoFisher Scientific, Hemel Hempstead, UK).

**GC-C-IRMS** isotope analyses of the various compound classes were carried out using a variety of instruments produced by ThermoFisher.

For C isotope analyses use was made of an instrument incorporating a Varian 3500 GC (Varian BV, Middelburg, The Netherlands) coupled to a Finnigan MAT DELTA S isotope ratio mass spectrometer (Finnigan MAT, Hemel Hempstead, UK). Analytes in He were combusted to CO<sub>2</sub> using a modified Type I Finnigan MAT combustion interface with a CuO/Pt combustion reactor set to a temperature of 850 °C prior to entry into the mass spectrometer source *via* an open split. The ionisation source electron energy was 100 eV with an electron current of 1 mA. Detection was *via* 3 Faraday cup collectors set at *m/z* 44, 45 and 46. The chromatographic conditions were the same as those described previously. All sample analyses were conducted in duplicate to ensure reliable  $\delta^{13}\text{C}$  values. Samples were calibrated against reference CO<sub>2</sub> of known isotopic composition which was introduced directly into the source three times at the beginning and end of every run. Compound specific IRMS performance was determined using a suite of externally calibrated reference fatty acid methyl esters. Samples were analysed with a precision <0.5 ‰ ( $\pm 1$  standard deviation, *n*=5).

The  $\delta^{15}\text{N}$  values of individual AAs were determined as their *N*-acetyl, *O*-isopropyl derivatives and were determined using a ThermoFinnigan Trace 2000 gas chromatograph coupled with a ThermoFinnigan DeltaPlus XP isotope ratio mass spectrometer *via* a ThermoFinnigan Combustion III Interface (Thermo Electron Corporation, Waltham, USA). Samples were introduced using a CTC Analytics GC Pal autosampler (Zwingen, Switzerland) and *via* a programmable temperature vaporisation (PTV) inlet (Thermo Electron Corporation, Waltham, USA). The carrier gas was helium (He; The BOC Group plc, Guildford, UK) at a flow rate of 1.4 mL minute<sup>-1</sup> and the gas chromatograph was fitted with a DB-35 column (30 m  $\times$  0.32 mm i.d.  $\times$  0.5  $\mu\text{m}$  stationary phase thickness; Agilent Technologies, Santa Clara, USA). The temperature programme utilised was: 40 °C (5 min) to 120 °C at 15 °C min<sup>-1</sup>, to 180 °C at 3 °C min<sup>-1</sup>, then to 210 °C at 1.5 °C min<sup>-1</sup> and finally to 270 °C at 5 °C min<sup>-1</sup>. The oxidation reactor comprised Cu, Ni and Pt wires (high purity from OEA Laboratories Ltd., Callington, UK) maintained at 980 °C; the reduction reactor comprised Cu wires maintained at 650 °C. AA  $\delta^{15}\text{N}$  values were determined relative to that of a monitoring gas of known (previously determined using in-house AA standards) N-isotopic composition introduced directly into the ion source *via* an open split in four pulses at the beginning and end of each run. The  $\delta^{15}\text{N}$  values of the in-house AA standards were determined off-line by elemental analysis-isotope ratio mass spectrometry (EA-IRMS) by ThermoFisher Scientific (Bremen, Germany) and by NERC CEH (Merlewood/Lancaster, UK) using primary reference materials (NIST 8547 IAEA-N-1 ammonium sulphate;  $\delta^{15}\text{N}$  +0.4 ‰). In order to adhere to the identical treatment principle and ensure the GC-C-IRMS system was functioning properly, each sample was bracketed by the in-house AA standard mixture of known  $\delta^{15}\text{N}$  values and sample AA  $\delta^{15}\text{N}$  values accepted only when at least 75 % of the AAs in the standard mixture run either side of the sample were within  $\pm 1$  ‰ and the others were within  $\pm 1.5$  ‰, and when this was also true on average over the course of the run. Data were acquired and analysed using Isodat NT 3.0 (ThermoFinnigan, Thermo Electron Corporation, Waltham, USA).

Samples in which the substrate AAs (or others) were significantly more enriched in <sup>15</sup>N or <sup>13</sup>C than endogenous AAs were analysed twice: once where the more enriched peaks were vented before entering the oxidation and reduction reactors (Fig. 2b in Knowles *et al.*, [2010]), in order to avoid possible reactor memory effects that may affect the  $\delta$  values of subsequent eluting peaks, and once where all compounds were allowed to pass through the reactors and enter the mass spectrometer in order to determine  $\delta$  values for the more enriched compounds (Fig. 2a in Knowles *et al.*, [2010]).

### Calculations for <sup>15</sup>N-SIP of AA

If the total hydrolysable AA pool is taken to be representative of the soil protein pool, then any <sup>15</sup>N enrichment (*E*) in hydrolysable AAs can be summed to represent newly synthesised soil protein in the soil at that time:

$$\text{Newly synthesised soil protein} \approx \sum E \text{ in hydrolysable AAs} \quad \text{Eq.1}$$

The  $E$  of an AA may be expressed as the number of moles of  $^{15}\text{N}$  derived from the applied substrate that are present in that AA in the soil:

$$E = n_{\text{N}} \times \text{AFE} \quad \text{Eq.2}$$

where  $n_{\text{N}}$  is the number of moles of N in the AA (i.e. if the molecular structure of the AA contains only one N atom,  $n_{\text{N}}$  is the same as the number of moles of the AA in the soil, but twice this if the AA structure consists of two N atoms and so on) and AFE is the atom fraction excess of the AA after incubation compared with the control:

$$\text{AFE} = \text{AF}_{\text{Sample}} - \text{AF}_{\text{Control}} \quad \text{Eq.3}$$

AF is the atom fraction of  $^{15}\text{N}$  in the AA, i.e.:

$$\frac{\text{Number } ^{15}\text{N atoms}}{(^{14}\text{N} + ^{15}\text{N}) \text{ atoms}} \quad \text{Eq.4}$$

This can be calculated from the AA's  $\delta^{15}\text{N}$  value as in Knowles *et al.* [2010]:

$$\text{AF} = \frac{R_{\text{Std}}(\delta^{15}\text{N}/1000 + 1)}{1 + (\delta^{15}\text{N}/1000 + 1)} \quad \text{Eq.5}$$

where  $R_{\text{Std}}$  is the  $^{15}\text{N}/^{14}\text{N}$  ratio of AIR, the international isotopic standard for N. The data may also be expressed in terms of the percentage of the applied  $^{15}\text{N}$  incorporated into each AA, as in Knowles *et al.* [2010]:

$$\% \text{ Incorporation} = \left( \frac{E}{N} \right) \times 100 \quad \text{Eq.6}$$

where  $N$  is the number of moles of excess  $^{15}\text{N}$  applied (above natural abundance). Percentage incorporations reflect both the concentration and  $^{15}\text{N}$  enrichment ( $\delta^{15}\text{N}$  value) of the AA (i.e. how much was incorporated if the AA at x concentration was  $^{15}\text{N}$ -enriched by x ‰) and the percentage of applied  $^{15}\text{N}$  incorporated into newly synthesised soil protein is determined by summing these results for individual AAs. Note that these percentage incorporation data will be affected by the conservation of applied  $^{15}\text{N}$  in the system, thus if  $^{15}\text{N}$  is lost from the system (e.g. over time), AA percentage incorporations may become skewed as there is less  $^{15}\text{N}$  available for incorporation than expected. The incubation experimental design described aims to limit any  $^{15}\text{N}$  losses from the system in order to obviate this issue, but it is equally possible to calculate percentage incorporations at time,  $t$ , based on the moles of applied  $^{15}\text{N}$  retained ( $N_{\text{R}}$ ; above natural abundance/control soil values) in the system at time,  $t$  (Equation 7), if bulk recovery of the applied  $^{15}\text{N}$  is low or decreases with time.

$$N_{\text{R}} = \text{AFE} \left( \frac{\% \text{ TN}}{1400} \right) \quad \text{Eq.7}$$



where AFE is the atom fraction excess of  $^{15}\text{N}$  in the bulk soil (calculated from bulk and control soil  $\delta^{15}\text{N}$  values using Equations 3 and 5) and % TN is the percentage total N content of the soil. Percentage retention of applied  $^{15}\text{N}$  was calculated as follows:

$$\% \text{ Retention } ^{15}\text{N} = \left( \frac{N_R}{N} \right) \times 100 \quad \text{Eq.8}$$

It can also be argued, however, that loss from the system is just another process competing against AA biosynthesis for N, so should not be discounted in this way. All of these approaches are valid and the most appropriate one will depend on the specifics of the experimental design and desired outcomes.

### Statistical analyses for SIS

Two approaches were taken to analyse the  $^{13}\text{C}$ -label incorporation curves. Linear and non-linear regressions were performed using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA), R 2.8.1 (R foundation), and Microsoft Excel (Microsoft, Redmond, WA, USA). Linear regression was applied to %-incorporation data from the first 9 d of the incubation to obtain initial zero-order rate constants, whereas separate non-linear regressions were applied to incorporation data from the first 50 d of the incubation before the switchover to unlabelled  $\text{CH}_4$ , and the C turnover data from 50 d to 150 d. The equations of the fitted lines were of the form:

$$\text{Incorporation:} \quad F = F^0 + (P - Y^0) * (1 - \exp(-K * t)) \quad \text{Eq.9}$$

$$\text{Decay:} \quad F = (F^0 - P) * \exp(-K * t) + P \quad \text{Eq.10}$$

Where F is the fraction of excess  $^{13}\text{C}$  incorporated into organic material, t is the incubation time in days,  $R^0$  is the initial R value at initial t, P is the plateau at maximum  $^{13}\text{C}$  incorporation (incorporation) or maximum  $^{13}\text{C}$  loss (decay) and K is the first order rate constant expressed in inverse days.

### Isotopic corrections for added derivative C

All  $\delta^{13}\text{C}$  values were corrected for derivatisation using a mass balance equation (11). The  $\delta^{13}\text{C}$  value of each batch of  $\text{BF}_3\text{-MeOH}$  was determined by bulk IRMS.

$$n_{cd}\delta^{13}C_{cd} = n_c\delta^{13}C_c + n_d\delta^{13}C_d \quad \text{Eq.11}$$

The fractional abundance (F) of  $^{13}\text{C}$  in the control ( $F_c$ ) and enriched ( $F_e$ ) PLFAs was used to calculate the concentration of  $^{13}\text{C}$  incorporated into PLFAs from the total PLFA concentration. The fractional abundance expresses the amount of  $^{13}\text{C}$  as a proportion of the total amount of carbon in the PLFA (12).

$$\begin{aligned} \text{Incorporation} &= (F_e - F_c)[\text{PLFA}]_e \\ F &= \frac{^{13}\text{C}}{^{13}\text{C} + ^{12}\text{C}} \end{aligned} \quad \text{Eq.12}$$

### Statistical analysis

Bacterial populations were compared by cluster analysis using SYSTAT Version 7.0. The distribution of PLFAs was used as a measure of similarity or distance. The analysis was performed on Euclidean distances between standardised data using averages.

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